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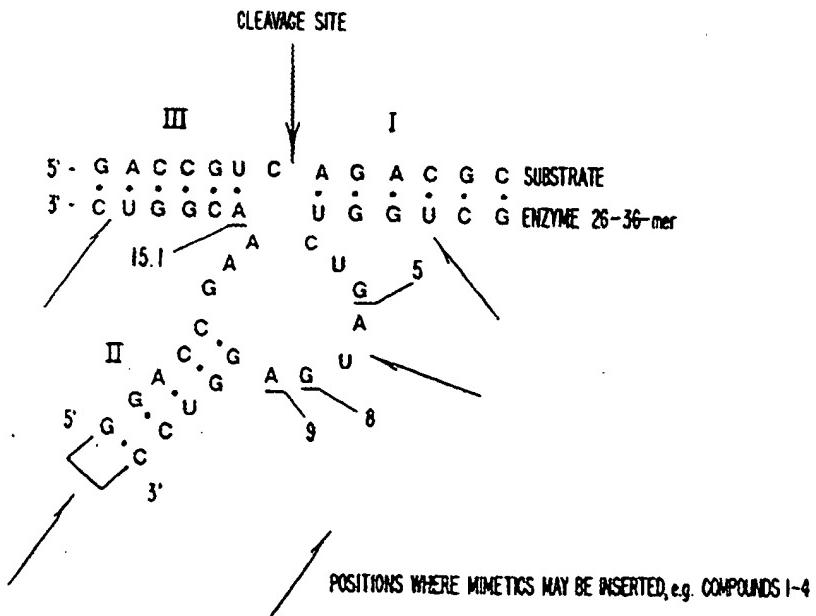


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(54) Title: NON-NUCLEOTIDE CONTAINING ENZYMATIC NUCLEIC ACID



(57) Abstract

Enzymatic nucleic acid molecule containing one or more non-nucleotide mimetics, and having activity to cleave an RNA or DNA molecule.

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DESCRIPTION

NON-NUCLEOTIDE CONTAINING ENZYMATIC NUCLEIC ACID

Background of the Invention

This application is a continuation-in-part of Usman et al., U.S. Serial No. 08/152,481, filed November 12, 1993 which is a continuation-in-part of Usman, U.S. 5 Serial No. 08/116,177, filed September 2, 1993, both entitled "Non-Nucleotide Containing Enzymatic Nucleic Acid" both hereby incorporated by reference herein (including drawings).

This invention relates to chemically synthesized 10 non-nucleotide-containing enzymatic nucleic acid.

The following is a brief history of the discovery and activity of enzymatic RNA molecules or ribozymes. This history is not meant to be complete but is provided only for understanding of the invention that 15 follows. This summary is not an admission that all of the work described below is prior art to the claimed invention.

Prior to the 1970s it was thought that all genes were direct linear representations of the proteins that 20 they encoded. This simplistic view implied that all genes were like ticker tape messages, with each triplet of DNA "letters" representing one protein "word" in the translation. Protein synthesis occurred by first transcribing a gene from DNA into RNA (letter for letter) 25 and then translating the RNA into protein (three letters at a time). In the mid 1970s it was discovered that some genes were not exact, linear representations of the proteins that they encode. These genes were found to contain interruptions in the coding sequence which were 30 removed from, or "spliced out" of, the RNA before it became translated into protein. These interruptions in

the coding sequence were given the name of intervening sequences (or introns) and the process of removing them from the RNA was termed splicing. At least three different mechanisms have been discovered for removing 5 introns from RNA. Two of these splicing mechanisms involve the binding of multiple protein factors which then act to correctly cut and join the RNA. A third mechanism involves cutting and joining of the RNA by the intron itself, in what was the first discovery of catalytic RNA 10 molecules.

Cech and colleagues were trying to understand how RNA splicing was accomplished in a single-celled pond organism called *Tetrahymena thermophila*. Cech proved that the intervening sequence RNA was acting as its own 15 splicing factor to snip itself out of the surrounding RNA. Continuing studies in the early 1980's served to elucidate the complicated structure of the *Tetrahymena* intron and to decipher the mechanism by which self-splicing occurs. Many research groups helped to demonstrate that the 20 specific folding of the *Tetrahymena* intron is critical for bringing together the parts of the RNA that will be cut and spliced. Even after splicing is complete, the released intron maintains its catalytic structure. As a consequence, the released intron is capable of carrying 25 out additional cleavage and splicing reactions on itself (to form intron circles). By 1986, Cech was able to show that a shortened form of the *Tetrahymena* intron could carry out a variety of cutting and joining reactions on other pieces of RNA. The demonstration proved that the 30 *Tetrahymena* intron can act as a true enzyme: (i) each intron molecule was able to cut many substrate molecules while the intron molecule remained unchanged, and (ii) reactions were specific for RNA molecules that contained a unique sequence (CUCU) which allowed the intron to 35 recognize and bind the RNA. Zaugg and Cech coined the term "ribozyme" to describe any ribonucleic acid molecule that has enzyme-like properties.

Also in 1986, Cech showed that the RNA substrate sequence recognized by the *Tetrahymena* ribozyme could be changed by altering a sequence within the ribozyme itself. This property has led to the development of a number of 5 site-specific ribozymes that have been individually designed to cleave at other RNA sequences.

The *Tetrahymena* intron is the most well-studied of what is now recognized as a large class of introns, Group I introns. The overall folded structure, including 10 several sequence elements, is conserved among the Group I introns, as is the general mechanism of splicing. Like the *Tetrahymena* intron, some members of this class are catalytic, i.e., the intron itself is capable of the self-splicing reaction. Other Group I introns require 15 additional (protein) factors, presumably to help the intron fold into and/or maintain its active structure.

Ribonuclease P (RNaseP) is an enzyme comprised of both RNA and protein components which are responsible for converting precursor tRNA molecules into their final 20 form by trimming extra RNA off one of their ends. RNaseP activity has been found in all organisms tested. Sidney Altman and his colleagues showed that the RNA component of RNaseP is essential for its processing activity; however, they also showed that the protein component also was 25 required for processing under their experimental conditions. After Cech's discovery of self-splicing by the *Tetrahymena* intron, the requirement for both protein and RNA components in RNaseP was reexamined. In 1983, Altman and Pace showed that the RNA was the enzymatic 30 component of the RNaseP complex. This demonstrated that an RNA molecule was capable of acting as a true enzyme, processing numerous tRNA molecules without itself undergoing any change.

The folded structure of RNaseP RNA has been 35 determined, and while the sequence is not strictly conserved between RNAs from different organisms, this higher order structure is. It is thought that the protein

component of the RNaseP complex may serve to stabilize the folded RNA *in vivo*.

Symons and colleagues identified two examples of a self-cleaving RNA that differed from other forms of catalytic RNA already reported. Symons was studying the propagation of the avocado sunblotch viroid (ASV), an RNA virus that infects avocado plants. Symons demonstrated that as little as 55 nucleotides of the ASV RNA was capable of folding in such a way as to cut itself into two pieces. It is thought that *in vivo* self-cleavage of these RNAs is responsible for cutting the RNA into single genome-length pieces during viral propagation. Symons discovered that variations on the minimal catalytic sequence from ASV could be found in a number of other plant pathogenic RNAs as well. Comparison of these sequences revealed a common structural design consisting of three stems and loops connected by a central loop containing many conserved (invariant from one RNA to the next) nucleotides. The predicted secondary structure for this catalytic RNA reminded the researchers of the head of a hammer; thus it was named as such.

Uhlenbeck was successful in separating the catalytic region of the ribozyme from that of the substrate. Thus, it became possible to assemble a hammerhead ribozyme from 2 (or 3) small synthetic RNAs. A 19-nucleotide catalytic region and a 24-nucleotide substrate were sufficient to support specific cleavage. The catalytic domain of numerous hammerhead ribozymes have now been studied by both the Uhlenbeck's and Symons' groups with regard to defining the nucleotides required for specific assembly and catalytic activity, and determining the rates of cleavage under various conditions.

Haseloff and Gerlach showed it was possible to divide the domains of the hammerhead ribozyme in a different manner. By doing so, they placed most of the required sequences in the strand that did not get cut (the

ribozyme) and only a required UH where H = C, A, or U in the strand that did get cut (the substrate). This resulted in a catalytic ribozyme that could be designed to cleave any UH RNA sequence embedded within a longer 5 "substrate recognition" sequence. The specific cleavage of a long mRNA, in a predictable manner using several such hammerhead ribozymes, was reported in 1988.

One plant pathogen RNA (from the negative strand of the tobacco ringspot virus) undergoes self-cleavage but 10 cannot be folded into the consensus hammerhead structure described above. Bruening and colleagues have independently identified a 50-nucleotide catalytic domain for this RNA. In 1990, Hampel and Tritz succeeded in dividing the catalytic domain into two parts that could 15 act as substrate and ribozyme in a multiple-turnover, cutting reaction. As with the hammerhead ribozyme, the catalytic portion contains most of the sequences required for catalytic activity, while only a short sequence (GUC in this case) is required in the target. Hampel and Tritz 20 described the folded structure of this RNA as consisting of a single hairpin and coined the term "hairpin" ribozyme (Bruening and colleagues use the term "paperclip" for this ribozyme motif). Continuing experiments suggest an increasing number of similarities between the hairpin and 25 hammerhead ribozymes in respect to both binding of target RNA and mechanism of cleavage.

Hepatitis Delta Virus (HDV) is a virus whose genome consists of single-stranded RNA. A small region (about 80 nucleotides) in both the genomic RNA, and in the 30 complementary anti-genomic RNA, is sufficient to support self-cleavage. In 1991, Been and Perrotta proposed a secondary structure for the HDV RNAs that is conserved between the genomic and anti-genomic RNAs and is necessary for catalytic activity. Separation of the HDV RNA into 35 "ribozyme" and "substrate" portions has recently been achieved by Been. Been has also succeeded in reducing the size of the HDV ribozyme to about 60 nucleotides.

The table below lists some of the characteristics of the ribozymes discussed above:

TABLE 1

Characteristics of Ribozymes

- 5 **Group I Introns**
Size: ~300 to >1000 nucleotides.
Requires a U in the target sequence immediately 5' of
the cleavage site.
Binds 4-6 nucleotides at 5' side of the cleavage
site.
10 Over 100 known members of this class. Found in
Tetrahymena thermophila rRNA, fungal mitochondria,
chloroplasts, phage T4, blue-green algae, and others.
RNaseP RNA (M1 RNA)
15 Size: ~290 to 400 nucleotides.
RNA portion of a ribonucleoprotein enzyme. Cleaves
tRNA precursors to form mature tRNA.
Roughly 10 known members of this group all are
bacterial in origin.
20 **Hammerhead Ribozyme**
Size: ~13 to 40 nucleotides.
Requires the target sequence UH immediately 5' of the
cleavage site.
Binds a variable number nucleotides on both sides of
25 the cleavage site.
14 known members of this class. Found in a number of
plant pathogens (virusoids) that use RNA as the
infectious agent. (Figure 1)
Hairpin Ribozyme
30 Size: ~50 nucleotides.
Requires the target sequence GUC immediately 3' of
the cleavage site.
Binds 4 nucleotides at 5' side of the cleavage site
and a variable number to the 3' side of the cleavage
site.
35 Only 1 known member of this class. Found in one
plant pathogen (satellite RNA of the tobacco ringspot

virus) which uses RNA as the infectious agent.
(Figure 2)

Hepatitis Delta Virus (HDV) Ribozyme

Size: ~60 nucleotides (at present).

- 5 Cleavage of target RNAs recently demonstrated.
Sequence requirements not fully determined.
Binding sites and structural requirements not fully
determined, although no sequences 5' of cleavage site
are required.
- 10 Only 1 known member of this class. Found in human
HDV. (Figure 3)

Eckstein et al., International Publication No.
WO 92/07065; Perrault et al., Nature 1990, 344:565; Pieken
et al., Science 1991, 253:314; Usman and Cedergren, Trends
15 in Biochem. Sci. 1992, 17:334; Usman et al., International
Publication No. WO 93/15187; and Rossi et al.;
International Publication No. WO 91/03162, describe
various chemical modifications that can be made to the
sugar moieties of enzymatic nucleic acid molecules.

20 Summary of the Invention

This invention concerns the use of
non-nucleotide molecules as spacer elements at the base of
double-stranded nucleic acid (e.g., RNA or DNA) stems
(duplex stems) or in the single-stranded regions,
25 catalytic core, loops, or recognition arms of enzymatic
nucleic acids. Duplex stems are ubiquitous structural
elements in enzymatic RNA molecules. To facilitate the
synthesis of such stems, which are usually connected via
single-stranded nucleotide chains, a base or base-pair
30 mimetic may be used to reduce the nucleotide requirement
in the synthesis of such molecules, and to confer nuclease
resistance (since they are non-nucleic acid components).
This also applies to both the catalytic core and
recognition arms of a ribozyme.

35 Examples of such non-nucleotide mimetics are
shown in Figure 4 and their incorporation into hammerhead

ribozymes is shown in Figure 5. These non-nucleotide linkers may be either polyether, polyamine, polyamide, or polyhydrocarbon compounds. Specific examples include those described by Seela and Kaiser, *Nucleic Acids Res.* 1990, 18:6353 and *Nucleic Acids Res.* 1987, 15:3113; Cload and Schepartz, *J. Am. Chem. Soc.* 1991, 113:6324; Richardson and Schepartz, *J. Am. Chem. Soc.* 1991, 113:5109; Ma et al., *Nucleic Acids Res.* 1993, 21:2585 and *Biochemistry* 1993, 32:1751; Durand et al., *Nucleic Acids Res.* 1990, 18:6353; McCurdy et al., *Nucleosides & Nucleotides* 1991, 10:287; Jäschke et al., *Tetrahedron Lett.* 1993, 34:301; Ono et al., *Biochemistry* 1991, 30:9914; Arnold et al., International Publication No. WO 89/02439 entitled "Non-nucleotide Linking Reagents for Nucleotide Probes"; and Ferentz and Verdine, *J. Am. Chem. Soc.* 1991, 113:4000, all hereby incorporated by reference herein.

Thus, in a first aspect, the invention features an enzymatic nucleic acid molecule having one or more non-nucleotide moieties, and having enzymatic activity to cleave an RNA or DNA molecule.

Examples of such non-nucleotide mimetics are shown in Figure 4 and their incorporation into hammerhead ribozymes is shown in Figure 5. These non-nucleotide linkers may be either polyether, polyamine, polyamide, or polyhydrocarbon compounds.

In preferred embodiments, the enzymatic nucleic acid includes one or more stretches of RNA, which provide the enzymatic activity of the molecule, linked to the non-nucleotide moiety.

By the term "non-nucleotide" is meant any group or compound which can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including either sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their enzymatic activity. The group or compound is abasic in that it does not contain a commonly recognized nucleotide base, such as

adenosine, guanine, cytosine, uracil or thymine. It may have substitutions for a 2' or 3' H or OH as described in the art. See Eckstein et al. and Usman et al., supra.

In preferred embodiments, the enzymatic nucleic acid includes one or more stretches of RNA, which provide the enzymatic activity of the molecule, linked to the non-nucleotide moiety. The necessary RNA components are known in the art, see, e.g., Usman, supra.

As the term is used in this application, non-nucleotide-containing enzymatic nucleic acid means a nucleic acid molecule that contains at least one non-nucleotide component which replaces a portion of a ribozyme, e.g., but not limited to, a double-stranded stem, a single-stranded "catalytic core" sequence, a single-stranded loop or a single-stranded recognition sequence. These molecules are able to cleave (preferably, repeatedly cleave) separate RNA or DNA molecules in a nucleotide base sequence specific manner. Such molecules can also act to cleave intramolecularly if that is desired. Such enzymatic molecules can be targeted to virtually any RNA transcript. Such molecules also include nucleic acid molecules having a 3' or 5' non-nucleotide, useful as a capping group to prevent exonuclease digestion.

Enzymatic molecules of this invention act by first binding to a target RNA or DNA. Such binding occurs through the target binding portion of the enzyme which is held in close proximity to an enzymatic portion of molecule that acts to cleave the target RNA or DNA. Thus, the molecule first recognizes and then binds a target nucleic acid through complementary base-pairing, and once bound to the correct site, acts enzymatically to cut the target. Strategic cleavage of such a target will destroy its ability to direct synthesis of an encoded protein. After an enzyme of this invention has bound and cleaved its target it is released from that target to search for

another target, and can repeatedly bind and cleave new targets.

The enzymatic nature of an enzyme of this invention is advantageous over other technologies, such as 5 antisense technology (where a nucleic acid molecule simply binds to a nucleic acid target to block its translation) since the effective concentration of the enzyme necessary to effect a therapeutic treatment is lower than that of an antisense oligonucleotide. This advantage reflects the 10 ability of the enzyme to act enzymatically. Thus, a single enzyme molecule is able to cleave many molecules of target RNA. In addition, the enzyme is a highly specific inhibitor, with the specificity of inhibition depending not only on the base pairing mechanism of binding, but 15 also on the mechanism by which the molecule inhibits the expression of the RNA to which it binds. That is, the inhibition is caused by cleavage of the target and so specificity is defined as the ratio of the rate of cleavage of the targeted nucleic acid over the rate of 20 cleavage of non-targeted nucleic acid. This cleavage mechanism is dependent upon factors additional to those involved in base pairing. Thus, it is thought that the specificity of action of an enzyme of this invention is greater than that of antisense oligonucleotide binding the 25 same target site.

By the phrase enzyme is meant a catalytic non-nucleotide-containing nucleic acid molecule that has complementarity in a substrate-binding region to a specified nucleic acid target, and also has an enzymatic 30 activity that specifically cleaves RNA or DNA in that target. That is, the enzyme is able to intramolecularly or intermolecularly cleave RNA or DNA and thereby inactivate a target RNA or DNA molecule. This complementarity functions to allow sufficient 35 hybridization of the enzymatic molecule to the target RNA or DNA to allow the cleavage to occur. One hundred

percent complementarity is preferred, but complementarity as low as 50-75% may also be useful in this invention.

In preferred embodiments of this invention, the enzyme molecule is formed generally in a hammerhead motif,
5 but may also be formed in the motif of a hairpin, hepatitis delta virus, group I intron or RNaseP RNA (in association with an RNA guide sequence). Examples of such hammerhead motifs are described by Rossi et al., *Aids Research and Human Retroviruses* 1992, 8:183; of hairpin
10 motifs by Hampel et al., "RNA Catalyst for Cleaving Specific RNA Sequences," filed September 20, 1989, which is a continuation-in-part of U.S. Serial No. 07/247,100 filed September 20, 1988, Hampel and Tritz, *Biochemistry* 1989, 28:4929, and Hampel et al., *Nucleic Acids Research*
15 1990, 18:299; and an example of the hepatitis delta virus motif is described by Perrotta and Been, *Biochemistry* 1992, 31:16; of the RNaseP motif by Guerrier-Takada et al., *Cell* 1983, 35:849; and of the Group I intron by Cech et al., U.S. Patent 4,987,071. These specific motifs are
20 not limiting in the invention and those skilled in the art will recognize that all that is important in an enzyme molecule of this invention is that it have at least one non-nucleotide portion, and a specific substrate-binding site which is complementary to one or more of the target
25 gene RNA regions, and that it have nucleotide sequences within or surrounding that substrate-binding site which impart a nucleic acid cleaving activity to the molecule.

The invention provides a method for producing a class of enzymatic cleaving agents which exhibit a high
30 degree of specificity for the nucleic acid of a desired target. The enzyme molecule is preferably targeted to a highly conserved sequence region of a target such that specific treatment of a disease or condition can be provided with a single enzyme. Such enzyme molecules can
35 be delivered exogenously to specific cells as required. In the preferred hammerhead motif the small size (less than 60 nucleotides, preferably between 30-40 nucleotides

in length) of the molecule allows the cost of treatment to be reduced compared to other ribozyme motifs.

Synthesis of nucleic acids greater than 100 nucleotides in length is difficult using automated methods, and the therapeutic cost of such molecules is prohibitive. In this invention, small enzyme motifs (e.g., of the hammerhead structure) are used for exogenous delivery. The simple structure of these molecules increases the ability of the enzyme to invade targeted regions of mRNA structure. Unlike the situation when the hammerhead structure is included within longer transcripts, there are no non-enzyme flanking sequences to interfere with correct folding of the enzyme structure or with complementary regions.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

The drawings will first briefly be described.

20 Drawings:

Figure 1 is a diagrammatic representation of the hammerhead ribozyme domain known in the art.

Figure 2 is a diagrammatic representation of the general structure of the hairpin ribozyme domain known in the art.

Figure 3 is a diagrammatic representation of the general structure of the hepatitis delta virus ribozyme domain known in the art.

Figure 4 is a diagrammatic representation of various non-nucleotide mimetics that may be incorporated into nucleic acid enzymes. Standard abbreviations are used in the Figure. In compound 1 each X may independently be oxygen, nitrogen, sulfur or substituted carbons containing alkyl, alkene or equivalent chains of length 1-10 carbon atoms. In compounds 6, 6a, 7, 8, 9 and 10 each Y may independently be a phosphodiester, ether or amide linkage to the rest of the nucleic acid enzyme. In

compounds 4 and 5 each R may independently be H, OH, protected OH, O-alkyl, alkenyl or alkynyl or alkyl, alkenyl or alkynyl of 1-10 carbon atoms.

Figure 5 is a diagrammatic representation of the 5 preferred location for incorporation of various non-nucleotide mimetics into nucleic acid enzymes. Specifically, mimetics, 1-10, may replace the loop (denoted as in Figure 5) that connects the two strands of Stem II. Stem II itself may be from 1 to 10 base pairs. In examples 1 & 2 below compounds 1 and 2 were incorporated into molecules having a stem II of 1 to 5 basepairs in length. Compounds 1, 4 and 5 may also replace nucleotides in the recognition arms of stems I and III or in stem II itself.

15 Figure 6 is a diagrammatic representation of the synthesis of a perylene based non-nucleotide mimetic phosphoramidite 3.

Figure 7 is a diagrammatic representation of the synthesis of an abasic deoxyribose or ribose 20 non-nucleotide mimetic phosphoramidite.

Figures 8a and 8b are graphical representations of cleavage of substrate by various ribozymes at 8nM, or 40 nM, respectively.

Non-nucleotide Mimetics

25 Non-nucleotide mimetics useful in this invention are generally described above. Those in the art will recognize that these mimetics can be incorporated into an enzymatic molecule by standard techniques at any desired location. Suitable choices can be made by standard 30 experiments to determine the best location, e.g., by synthesis of the molecule and testing of its enzymatic activity. The optimum molecule will contain the known ribonucleotides needed for enzymatic activity, and will have non-nucleotides which change the structure of the 35 molecule in the least way possible. What is desired is that several nucleotides can be substituted by one non-nucleotide to save synthetic steps in enzymatic molecule

synthesis and to provide enhanced stability of the molecule compared to RNA or even DNA.

Examples

The following are non-limiting examples showing
5 the synthesis of non-nucleotide mimetic-containing catalytic nucleic acids using non-nucleotide phosphoramidites.

Example 1: Synthesis of Hammerhead Ribozymes Containing Non-nucleotide Mimetics: Polyether Spacers

Polyether spacers, compound 1 (Figure 4; X=0, n=2 or 4), have been incorporated both singly, n=2 or 4, or doubly, n=2, at the base of stem II of a hammerhead ribozyme, replacing loop 2, and shown to produce a ribozyme which has lower catalytic efficiency. The method
10 of synthesis used followed the procedure for normal RNA synthesis as described in Usman et al., J. Am. Chem. Soc. 1987, 109:7845 and in Scaringe et al., Nucleic Acids Res. 1990, 18:5433, and makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at
15 the 5'-end, and phosphoramidites at the 3'-end. The design of these types of mimetics has not been optimized to date, but, as discussed above, this can be readily achieved using standard experimental techniques. These experiments
20 indicate the potential of such mimetics to replace the loops and portions of stems in ribozymes while maintaining catalytic activity. These mimetics may be incorporated not only into hammerhead ribozymes, but also into hairpin, hepatitis delta virus, or Group 1 or Group 2 introns.
25 They are, therefore, of general use as replacement motifs in any nucleic acid structure. Use of such mimetics allows about 2-10 nucleotides to be omitted from the final nucleic acid molecule compared to the use of an oligonucleotide without a non-nucleotide mimetic.

30 35 Example 2: Synthesis of Hammerhead Ribozymes Containing Non-nucleotide Mimetics: Aromatic Spacers

In another example, a specific linker for the base of the stem II C-G of a hammerhead ribozyme was designed. Applicant believes that the distance between the C1' carbons of the C-G base pair is about 16
5 Angstroms. To join these two pieces of RNA by a covalent analog of the C-G base pair a new type of dimer phosphoramidite containing a linker between the 3'-OH and the 5'-OH of the G and C residues respectively can be constructed. Two types of base-pair mimetic are the rigid
10 aromatic spacers, 2 or 3, shown in Figure 4. These have been incorporated at the base of stem II of a hammerhead ribozyme as described in Example 1, replacing loop 2, and shown to produce a ribozyme which has lower catalytic efficiency. Another mimetic is a flexible alkyl spacer
15 similar to the polyamide backbone described by Nielsen et al., Science 1991, 254:1497 (see, Figure 4; 6 or a derivative thereof 6a; Zuckerman et al., J. Am. Chem. Soc. 1992, 114:10464). Use of such mimetics allows about 2-10 nucleotides to be omitted from the final nucleic acid
20 molecule compared to the use of an oligonucleotide without a non-nucleotide mimetic.

Example 3: Synthesis of Non-nucleotide Mimetics Aromatic Spacer Phosphoramidite 2

This compound was originally described by
25 Salunkhe et al., J. Am. Chem. Soc. 1992, 114:6324. The synthesis was modified as follows: To terphthalic acid (1.0 g, 6.0 mmol) in DMF (12 mL) was added EDC (2.54 g, 13.2 mmol), aminohexanol (1.55 g, 13.2 mmol) and N-methylmorpholine (1.45 mL, 13.2 mmol). The reaction
30 mixture was stirred overnight at which time the solution was cloudy. Water was added to the reaction mixture to precipitate out the product. The solid was filtered and washed with water and dried to provide 562 mg (25.7%) of the diol.

35 To the diol (250 mg, 0.687 mmol) in DMSO (40 mL) was added triethylamine (287 μ L, 2.06 mmol), dimethoxytrityl chloride (220 mg, 0.653 mmol) and

catalytic DMAP. The reaction mixture was heated to 40°C and stirred overnight. The mixture was then cooled to room temperature (about 20-25°C), quenched with water and extracted three times with EtOAc. A solid precipitate 5 remained in the organic layer that was isolated and found to be starting diol (50 mg, 20%). The organic layer was dried over Na₂SO₄ and evaporated. The resulting oil was purified with flash chromatography (10% EtOAc in hexanes to 100% EtOAc) to yield 250 mg (55%) of the monotriylated 10 compound.

To the alcohol (193 mg, 0.29 mmol) in THF (1 mL) at 0°C was added diisopropylethylamine (101 µL, 0.58 mmol) and then 2-cyanoethyl N,N-diisopropylamino chlorophosphoramide (78 µL, 0.35 mmol) dropwise. The 15 resulting mixture was stirred for 5 minutes and then warmed to room temperature. After 1 hour the reaction mixture was quenched with methanol and evaporated. The resulting oil was purified by flash chromatography (1:1 hexanes:EtOAc) to yield 158 mg (63%) of the phosphoramidite. 20

Example 4: Synthesis of Non-nucleotide Mimetics Aromatic Spacer Phosphoramidite 3

Referring to Figure 6, to 3, 4, 9, 10-pylenetetracarboxylic dianhydride 11 (1.0 g, 2.55 mmol) 25 in quinoline (10 mL) was added ethanolamine (919 µL, 15.3 mmol) and ZnOAc•2.5 H₂O (140 mg, 0.638 mmol). The reaction mixture was heated to 190°C for 8 hours. The solution was then cooled, 1N HCl added to precipitate the product and the mixture was filtered. The solid was washed with hot 30 10% CaCO₃ until the filtrate was no longer pale green. The remaining bright red precipitate 12 was then dried.

The resulting diol 12 was then treated as outlined above for 2 to provide the phosphoramidite 3.

Example 5: Synthesis of Hammerhead Ribozymes Containing 35 Non-nucleotide Mimetics: Abasic Nucleotides 4 and 5

Compound 4, R=H, was prepared according to Iyer et al., *Nucleic Acids Res.* 1990, 18:2855. Referring to Figure 7, compounds 4 and 5 (R=O-t-butylidimethylsilyl) phosphoramidites were prepared as follows:

5 To a solution of D-ribose (20.0 g, 0.105 mol) in N,N-dimethylformamide (250 mL) was added 2,2-dimethoxypropane (50 mL) and p-toluenesulfonic acid monohydrate (300 mg). The reaction mixture was stirred for 16 hours at room temperature and then evaporated to dryness. The crude product was coevaporated with pyridine (2 x 150 mL), dissolved in dry pyridine (300 mL) and 4,4'-dimethoxytrityl chloride (37.2 g, 0.110 mol) was added and stirred for 24 hours at room temperature. The reaction mixture was diluted with methanol (50 mL) and evaporated to dryness. The residue was dissolved in chloroform (800 mL) and washed with 5% NaHCO₃ (2 x 200 mL), brine (300 mL), dried, evaporated, coevaporated with toluene (2 x 100 mL) and purified by flash chromatography in CHCl₃ to yield 40.7 g (78.1%) of compound a.

20 To a solution of dimethoxytrityl derivative a (9.0 g, 18.3 mmol) and DMAP (4.34 g, 36 mmol) in dry CH₃CN, phenoxythiocarbonyl chloride (3.47 g, 20.1 mmol) was added dropwise under argon. The reaction mixture was left for 16 hours at room temperature, then evaporated to dryness. 25 The resulting residue was dissolved in chloroform (200 mL), washed with 5% NaHCO₃, brine, dried, evaporated and purified by flash chromatography in CHCl₃, to yield 8.0 g (69.5%) of compound b as the β -anomer.

To a solution of intermediate b (3.0 g, 4.77 mmol) in toluene (50 mL) was added AIBN (0.82 g, 5.0 mmol) and Bu₃SnH (1.74 g, 6.0 mmol) under argon and the reaction mixture was kept at 80°C for 7 hours. The solution was evaporated and the resulting residue purified by flash chromatography in CHCl₃, to yield 1.5 g (66%) of protected 35 ribitol c.

Subsequent removal of all protecting groups by acid treatment and tritylation provided the protected

ribitol d which was then converted to target phosphoramidites 4 and 5 by the general method described in Scaringe et al., *Nucleic Acids Res.* 1990, 18:5433.

Example 6

Referring to Figures 8a and 8b the cleavage of substrate is shown by various modified ribozymes compared to unmodified ribozyme at 8nM and 40nM concentrations. Specifically, a control ribozyme of sequence ucuccA UCU GAU GAG GCC GAA AGG CCG AAA Auc ccU (where lower case includes a 2' O-methyl group) was compared to ribozyme A (ucu ccA UCU GAU GAG GCC SGG CCG AAA Auc ccu), B (ucu ccA UCU GAU GAG CSG CG AAA Auc ccu), C (ucu ccA UCU GAU GAG GCC bbb bGG CCG AAA Auc ccu), and D (ucu ccA UCU GAU GAG Cbb bbG CGAA AAu ccc u) (where S=hexaethylene glycol linker); and b=abasic nucleotide 4). All were active in cleaving substrate.

Administration of Ribozyme

Selected ribozymes can be administered prophylactically, to viral infected patients or to diseased patients, e.g., by exogenous delivery of the ribozyme to a relevant tissue by means of an appropriate delivery vehicle, e.g., a liposome, a controlled release vehicle, by use of iontophoresis, electroporation or ion paired molecules, or covalently attached adducts, and other pharmacologically approved methods of delivery. Routes of administration include intramuscular, aerosol, oral (tablet or pill form), topical, systemic, ocular, intraperitoneal and/or intrathecal.

The specific delivery route of any selected ribozyme will depend on the use of the ribozyme. Generally, a specific delivery program for each ribozyme will focus on unmodified ribozyme uptake with regard to intracellular localization, followed by demonstration of efficacy. Alternatively, delivery to these same cells in an organ or tissue of an animal can be pursued. Uptake studies will include uptake assays to evaluate cellular ribozyme uptake, regardless of the delivery vehicle or

strategy. Such assays will also determine the intracellular localization of the ribozyme following uptake, ultimately establishing the requirements for maintenance of steady-state concentrations within the 5 cellular compartment containing the target sequence (nucleus and/or cytoplasm). Efficacy and cytotoxicity can then be tested. Toxicity will not only include cell viability but also cell function.

Some methods of delivery that may be used 10 include:

- a. encapsulation in liposomes,
- b. transduction by retroviral vectors,
- c. conjugation with cholesterol;
- d. localization to nuclear compartment utilizing antigen binding or nuclear targeting site found on most snRNAs or nuclear proteins,
- e. neutralization of charge of ribozyme by using nucleotide derivatives, and
- f. use of blood stem cells to distribute ribozymes throughout the body.

Delivery strategies useful in the present invention, include: ribozyme modifications, and particle carrier drug delivery vehicles. Unmodified ribozymes, 25 like most small molecules, are taken up by cells, albeit slowly. To enhance cellular uptake, the ribozyme may be modified essentially at random, in ways which reduce its charge but maintains specific functional groups. This results in a molecule which is able to diffuse across the 30 cell membrane, thus removing the permeability barrier.

Modification of ribozymes to reduce charge is just one approach to enhance the cellular uptake of these larger molecules. The random approach, however, is not advisable since ribozymes are structurally and 35 functionally more complex than small drug molecules. The structural requirements necessary to maintain ribozyme catalytic activity are well understood by those in the

art. These requirements are taken into consideration when designing modifications to enhance cellular delivery. The modifications are also designed to reduce susceptibility to nuclease degradation. Both of these characteristics 5 should greatly improve the efficacy of the ribozyme. Cellular uptake can be increased by several orders of magnitude without having to alter the phosphodiester linkages necessary for ribozyme cleavage activity.

Chemical modifications of the phosphate backbone 10 will reduce the negative charge allowing free diffusion across the membrane. This principle has been successfully demonstrated for antisense DNA technology. The similarities in chemical composition between DNA and RNA make this a feasible approach. In the body, maintenance 15 of an external concentration will be necessary to drive the diffusion of the modified ribozyme into the cells of the tissue. Administration routes which allow the diseased tissue to be exposed to a transient high concentration of the drug, which is slowly dissipated by 20 systemic adsorption are preferred. Intravenous administration with a drug carrier designed to increase the circulation half-life of the ribozyme can be used. The size and composition of the drug carrier restricts rapid clearance from the blood stream. The carrier, made 25 to accumulate at the site of infection, can protect the ribozyme from degradative processes.

Drug delivery vehicles are effective for both systemic and topical administration. They can be designed to serve as a slow release reservoir, or to deliver their 30 contents directly to the target cell. An advantage of using direct delivery drug vehicles is that multiple molecules are delivered per uptake. Such vehicles have been shown to increase the circulation half-life of drugs which would otherwise be rapidly cleared from the blood 35 stream. Some examples of such specialized drug delivery vehicles which fall into this category are liposomes,

hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres.

From this category of delivery systems, liposomes are preferred. Liposomes increase intracellular 5 stability, increase uptake efficiency and improve biological activity.

Liposomes are hollow spherical vesicles composed of lipids arranged in a similar fashion as those lipids which make up the cell membrane. They have an internal 10 aqueous space for entrapping water soluble compounds and range in size from 0.05 to several microns in diameter. Several studies have shown that liposomes can deliver RNA to cells and that the RNA remains biologically active.

For example, a liposome delivery vehicle 15 originally designed as a research tool, Lipofectin, has been shown to deliver intact mRNA molecules to cells yielding production of the corresponding protein. In another study, an antibody targeted liposome delivery system containing an RNA molecule 3,500 nucleotides in 20 length and antisense to a structural protein of HIV, inhibited virus proliferation in a sequence specific manner. Not only did the antibody target the liposomes to the infected cells, but it also triggered the internalization of the liposomes by the infected cells. 25 Triggering the endocytosis is useful for viral inhibition. Finally, liposome delivered synthetic ribozymes have been shown to concentrate in the nucleus of H9 (an example of an HIV-sensitive cell) cells and are functional as evidenced by their intracellular cleavage of the sequence. 30 Liposome delivery to other cell types using smaller ribozymes (less than 142 nucleotides in length) exhibit different intracellular localizations.

Liposomes offer several advantages: They are non-toxic and biodegradable in composition; they display 35 long circulation half-lives; and recognition molecules can be readily attached to their surface for targeting to tissues. Finally, cost effective manufacture of liposome-

based pharmaceuticals, either in a liquid suspension or lyophilized product, has demonstrated the viability of this technology as an acceptable drug delivery system.

Other controlled release drug delivery systems, such as nonoparticles and hydrogels may be potential delivery vehicles for a ribozyme. These carriers have been developed for chemotherapeutic agents and protein-based pharmaceuticals, and consequently, can be adapted for ribozyme delivery.

Topical administration of ribozymes is advantageous since it allows localized concentration at the site of administration with minimal systemic adsorption. This simplifies the delivery strategy of the ribozyme to the disease site and reduces the extent of toxicological characterization. Furthermore, the amount of material to be applied is far less than that required for other administration routes. Effective delivery requires the ribozyme to diffuse into the infected cells. Chemical modification of the ribozyme to neutralize negative charge may be all that is required for penetration. However, in the event that charge neutralization is insufficient, the modified ribozyme can be co-formulated with permeability enhancers, such as Azone or oleic acid, in a liposome. The liposomes can either represent a slow release presentation vehicle in which the modified ribozyme and permeability enhancer transfer from the liposome into the infected cell, or the liposome phospholipids can participate directly with the modified ribozyme and permeability enhancer in facilitating cellular delivery. In some cases, both the ribozyme and permeability enhancer can be formulated into a suppository formulation for slow release.

Ribozymes may also be systemically administered. Systemic absorption refers to the accumulation of drugs in the blood stream followed by distribution throughout the entire body. Administration routes which lead to systemic absorption include: intravenous, subcutaneous,

- intraperitoneal, intranasal, intrathecal and ophthalmic. Each of these administration routes expose the ribozyme to an accessible diseased tissue. Subcutaneous administration drains into a localized lymph node which
- 5 proceeds through the lymphatic network into the circulation. The rate of entry into the circulation has been shown to be a function of molecular weight or size. The use of a liposome or other drug carrier localizes the ribozyme at the lymph node. The ribozyme can be modified
- 10 to diffuse into the cell, or the liposome can directly participate in the delivery of either the unmodified or modified ribozyme to the cell. This method is particularly useful for treating AIDS using anti-HIV ribozymes.
- 15 Also preferred in AIDS therapy is the use of a liposome formulation which can deliver oligonucleotides to lymphocytes and macrophages. This oligonucleotide delivery system inhibits HIV proliferation in infected primary immune cells. Whole blood studies show that the
- 20 formulation is taken up by 90% of the lymphocytes after 8 hours at 37°C. Preliminary biodistribution and pharmacokinetic studies yielded 70% of the injected dose/gm of tissue in the spleen after one hour following intravenous administration. This formulation offers an
- 25 excellent delivery vehicle for anti-AIDS ribozymes for two reasons. First, T-helper lymphocytes and macrophages are the primary cells infected by the virus, and second, a subcutaneous administration delivers the ribozymes to the resident HIV-infected lymphocytes and macrophages in the
- 30 lymph node. The liposomes then exit the lymphatic system, enter the circulation, and accumulate in the spleen, where the ribozyme is delivered to the resident lymphocytes and macrophages.
- Intraperitoneal administration also leads to
- 35 entry into the circulation, with once again, the molecular weight or size of the ribozyme-delivery vehicle complex controlling the rate of entry.

Liposomes injected intravenously show accumulation in the liver, lung and spleen. The composition and size can be adjusted so that this accumulation represents 30% to 40% of the injected dose.

5 The remaining dose circulates in the blood stream for up to 24 hours.

The chosen method of delivery should result in cytoplasmic accumulation in the afflicted cells and molecules should have some nuclease-resistance for optimal 10 dosing. Nuclear delivery may be used but is less preferable. Most preferred delivery methods include liposomes (10-400 nm), hydrogels, controlled-release polymers, microinjection or electroporation (for ex vivo treatments) and other pharmaceutically applicable 15 vehicles. The dosage will depend upon the disease indication and the route of administration but should be between 100-200 mg/kg of body weight/day. The duration of treatment will extend through the course of the disease symptoms, usually at least 14-16 days and possibly 20 continuously. Multiple daily doses are anticipated for topical applications, ocular applications and vaginal applications. The number of doses will depend upon disease delivery vehicle and efficacy data from clinical trials.

25 Establishment of therapeutic levels of ribozyme within the cell is dependent upon the rate of uptake and degradation. Decreasing the degree of degradation will prolong the intracellular half-life of the ribozyme. Thus, chemically modified ribozymes, e.g., with 30 modification of the phosphate backbone, or capping of the 5' and 3' ends of the ribozyme with nucleotide analogues may require different dosaging. Descriptions of useful systems are provided in the art cited above, all of which is hereby incorporated by reference herein.

35 For a more detailed description of ribozyme design, see, Draper, U.S. Serial No. 08/103,243 filed

25

August 6, 1993, hereby incorporated by reference herein in its entirety.

Other embodiments are within the following claims.

"Sequence Listing"**(1) GENERAL INFORMATION:**

(i) APPLICANT: Nassim Usman
Francine E. Wincott
Jasenka Matulic-Adamic
Leonid Beigelman

(ii) TITLE OF INVENTION: NON-NUCLEOTIDE CONTAINING ENZYMATIC NUCLEIC ACID

(iii) NUMBER OF SEQUENCES: 5

(iv) CORRESPONDENCE ADDRESS:

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb storage
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: IBM P.C. DOS (Version 5.0)
(D) SOFTWARE: WordPerfect (Version 5.1)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

Prior applications total,
including application
described below: two

(A) APPLICATION NUMBER: 08/152,488
(B) FILING DATE: 12 NOV 1993

(A) APPLICATION NUMBER: 08/116,177
(B) FILING DATE: 02 SEPT 1993

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(C) REFERENCE/DOCKET NUMBER: 206/267

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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	11
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(ix) FEATURE:

(D) OTHER INFORMATION:	The letter "N" stands for any base. "H" represents nucleotide C, A, or U.
------------------------	---

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

NNNNUHNNNN N 11

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

46X	(A) LENGTH:	32
	(B) TYPE:	nucleic acid
	(C) STRANDEDNESS:	single
	(D) TOPOLOGY:	linear

(ix) FEATURE:

(D) OTHER INFORMATION:	The letter "N" stands for any base.
------------------------	-------------------------------------

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

NNNNNCUGAN GAGGCCGAAA GGCGAAANN NN 32

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	14
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(ix) FEATURE:

(D) OTHER INFORMATION:	The letter "N" stands for any base.
------------------------	-------------------------------------

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

NNNNNGUCNN NNNN

14

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	50
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(ix) FEATURE:

(D) OTHER INFORMATION:	The letter "N" stands for any base.
------------------------	-------------------------------------

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

NNNNNNAGAA NNNNACCAGA GAAACACACG UUGUGGUUAUUAUACCUGGUA

50

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	85
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

29

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

UGGCCGGCAU GGUCCCAGCC UCCUCGCUGG CGCCGGCUGG GCAACAUUCC 50
GAGGGGACCG UCCCCUCGGU AAUGGCGAAU GGGAC 85

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 36
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

UCUCCAUCUG AUGAGGCCGA AAGGCCGAAA AUCCU 36

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

UCUCCAUCUG AUGAGGCCSG GCCGAAAAUC CCU 33

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

UCUCCAUCUG AUGAGCAGCG AAAAUCCU 29

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

30

(A) LENGTH:	36
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

UCUCCAUCUG AUGAGGCCBB BBGGCCGAAA AUCCU

36.

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	32
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

UCUCCAUCUG AUGAGCBBBB GCGAAAAUCC CU

32.

Claims

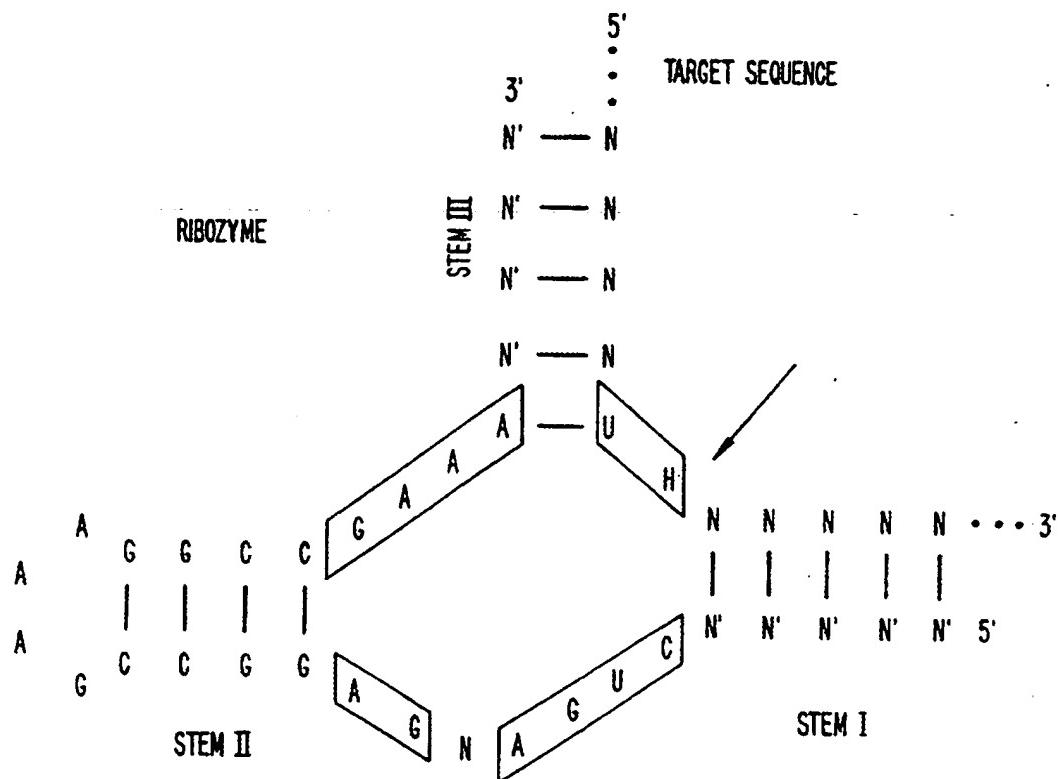
1. Enzymatic nucleic acid comprising a non-nucleotide.

2. The enzymatic nucleic acid of claim 1, wherein
5 said non-nucleotide is provided in a double-stranded stem
region, the catalytic core, or in a single-stranded
recognition region.

3. The enzymatic nucleic acid of claim 1, wherein
said non-nucleotide is selected from the group consisting
10 of compound 1, 2, 3, 4 and 5.

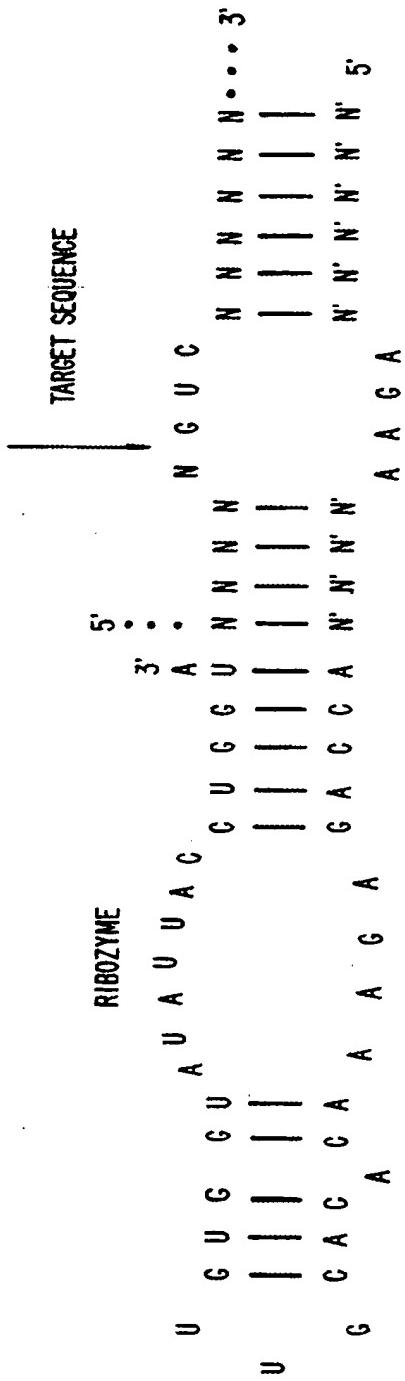
1/9

FIG. I.



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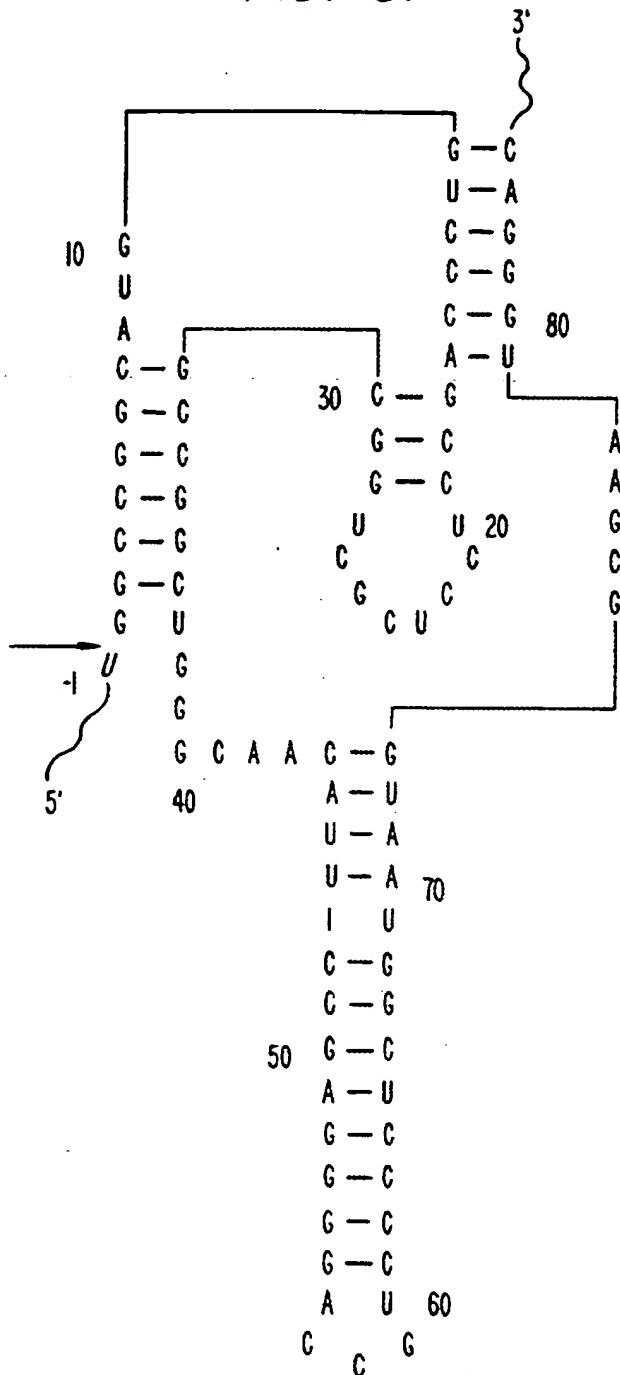
FIG. 2.



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FIG. 3.



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FIG. 4. SPACERS

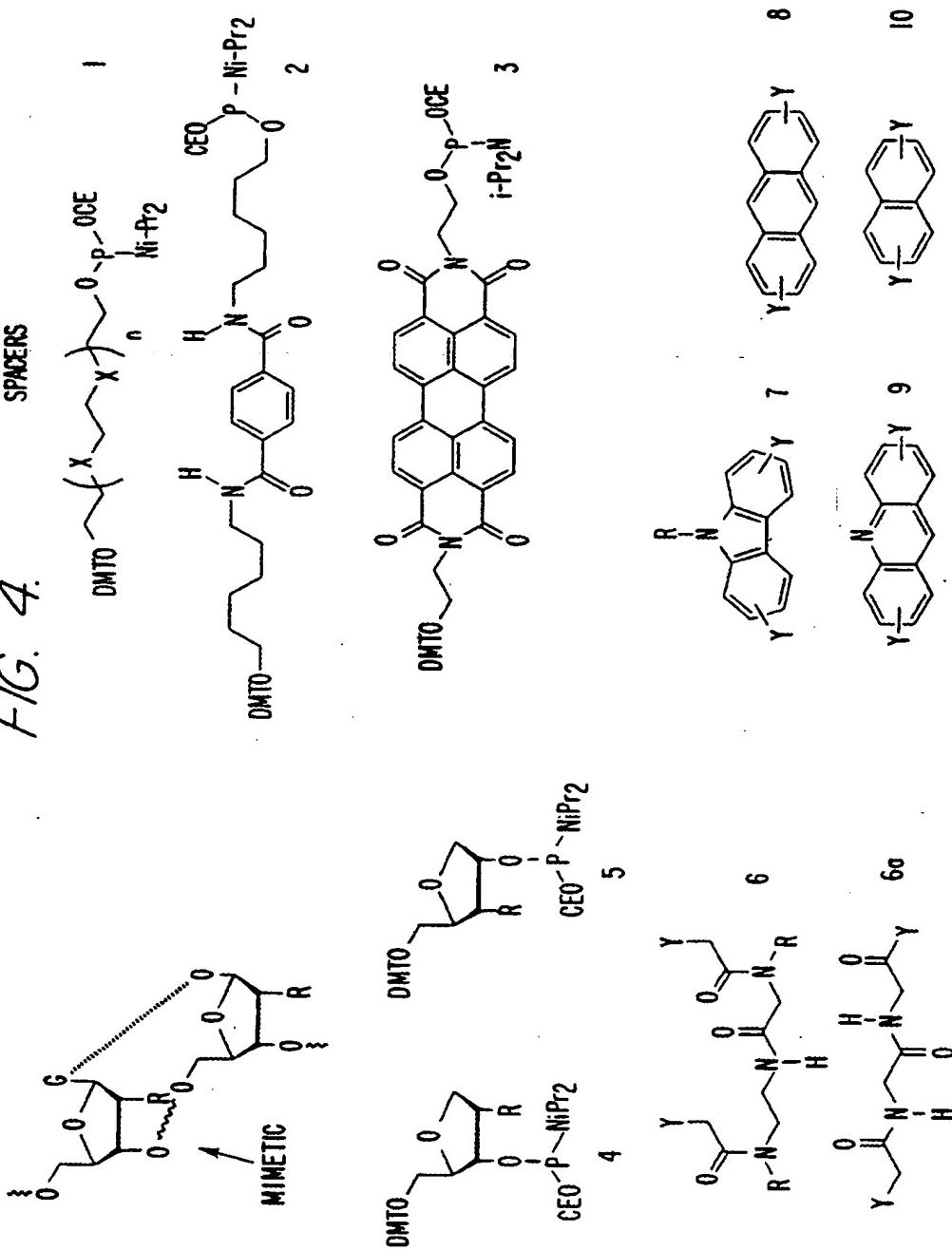
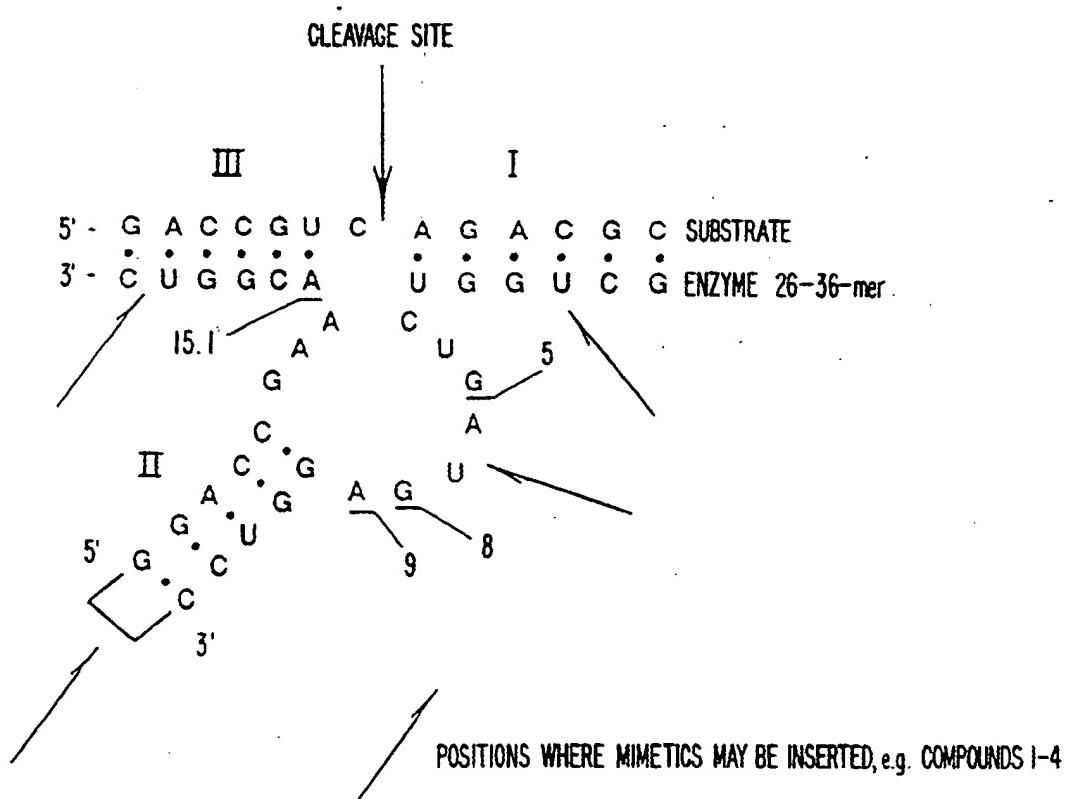
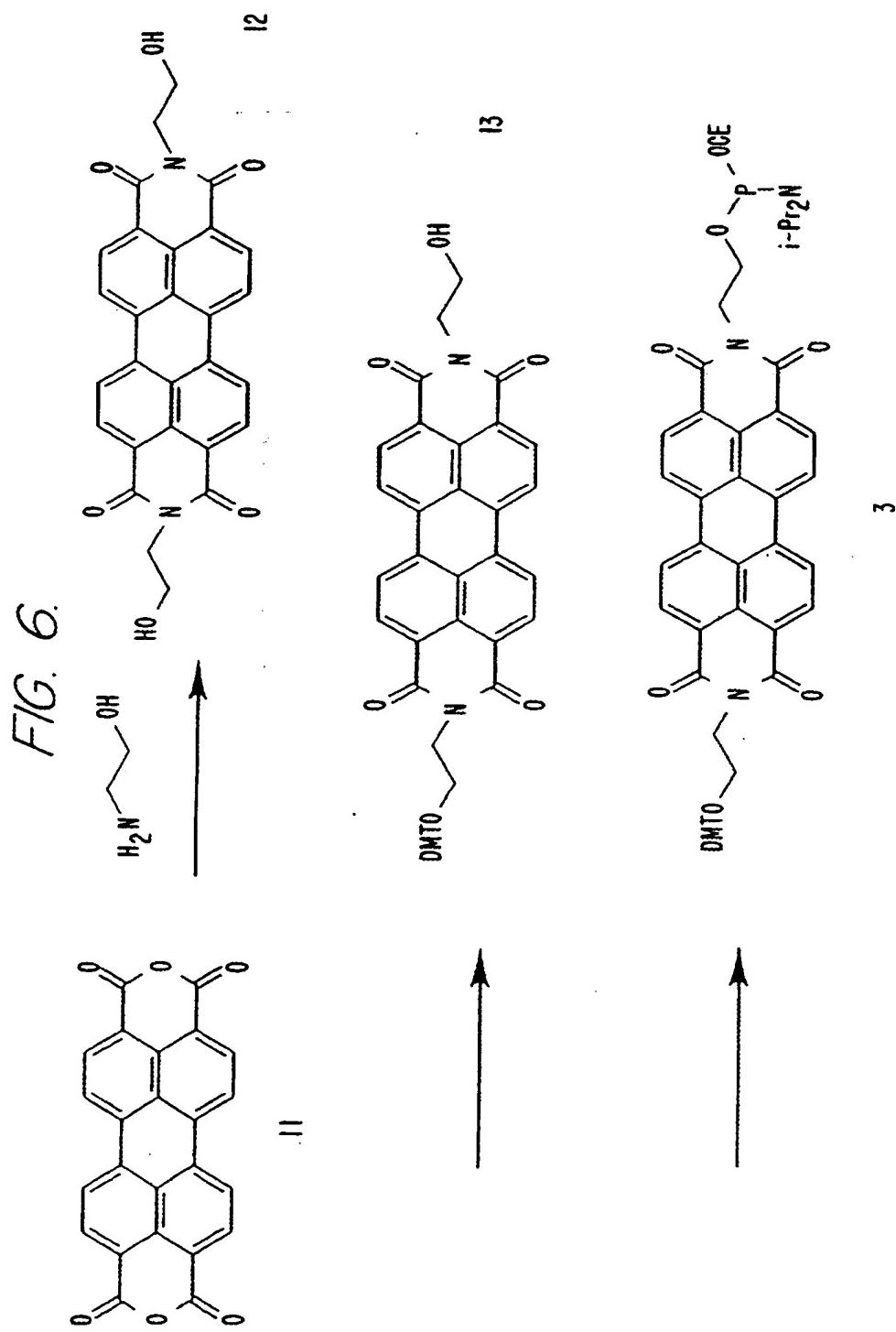


FIG. 5.



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FIG. 7.

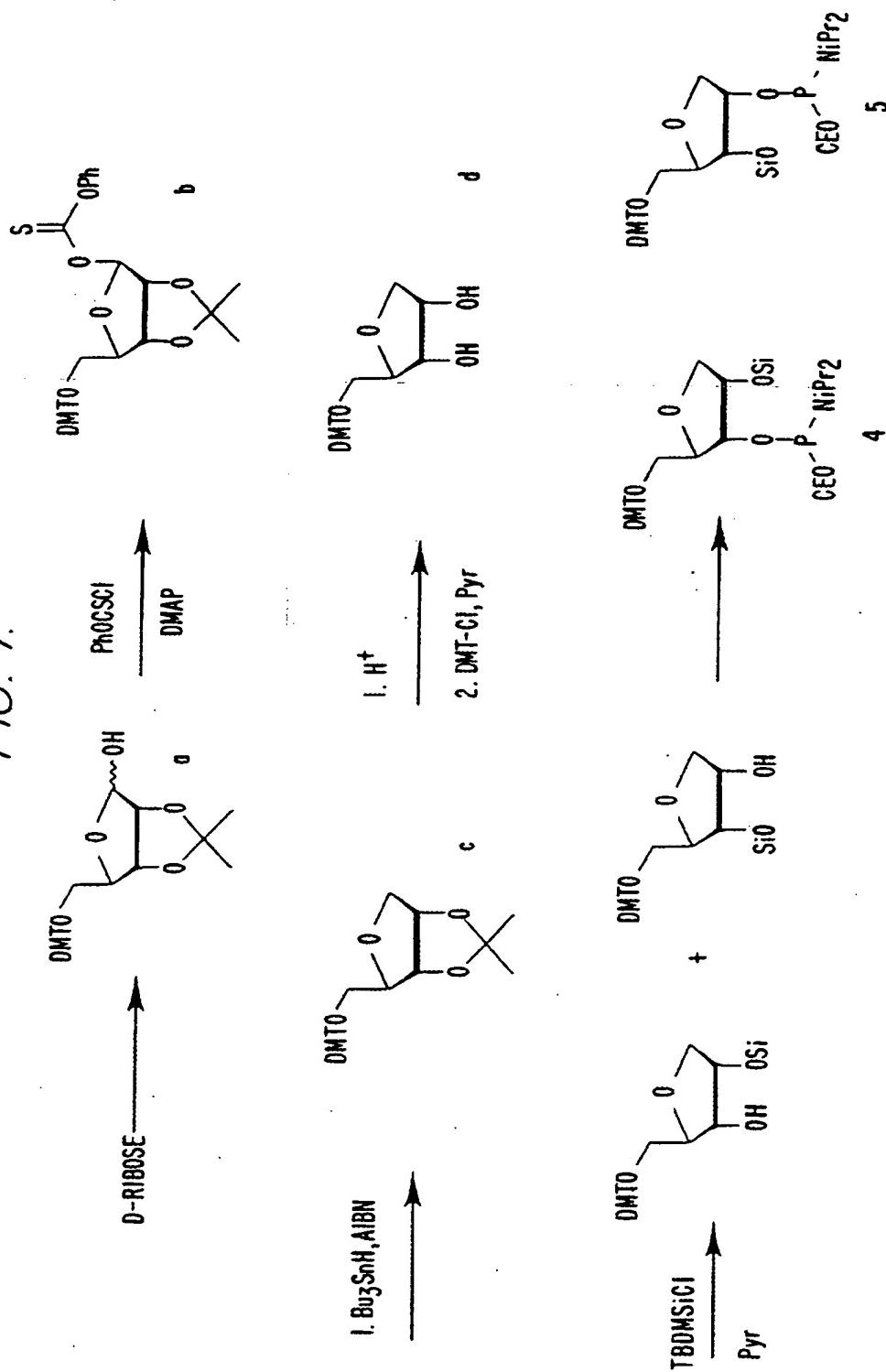
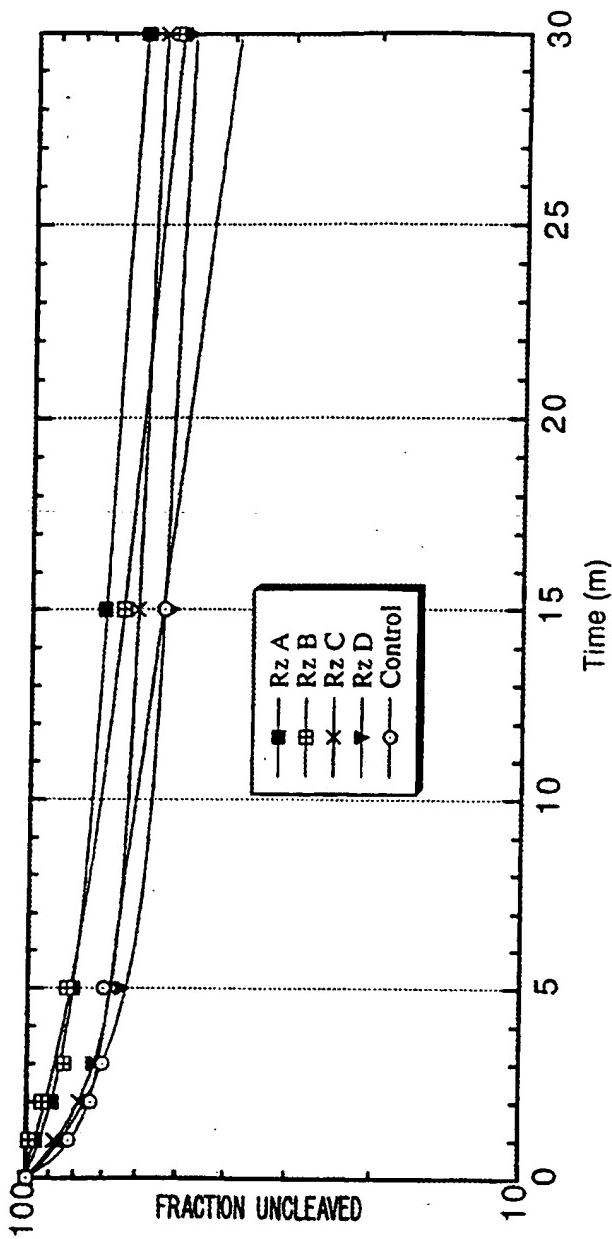


FIG. 8a.



$Rz A$		
$y = m1 \cdot \exp(-m2 \cdot m0) + m3 \cdot \exp(-m4 \cdot m0)$		
	Value	Error
m1	18.469	3.7629
m2	0.39253	0.1409
m3	82.388	3.7527
m4	0.010447	0.0020647
Chisq	7.4368	NA
R	0.99682	NA

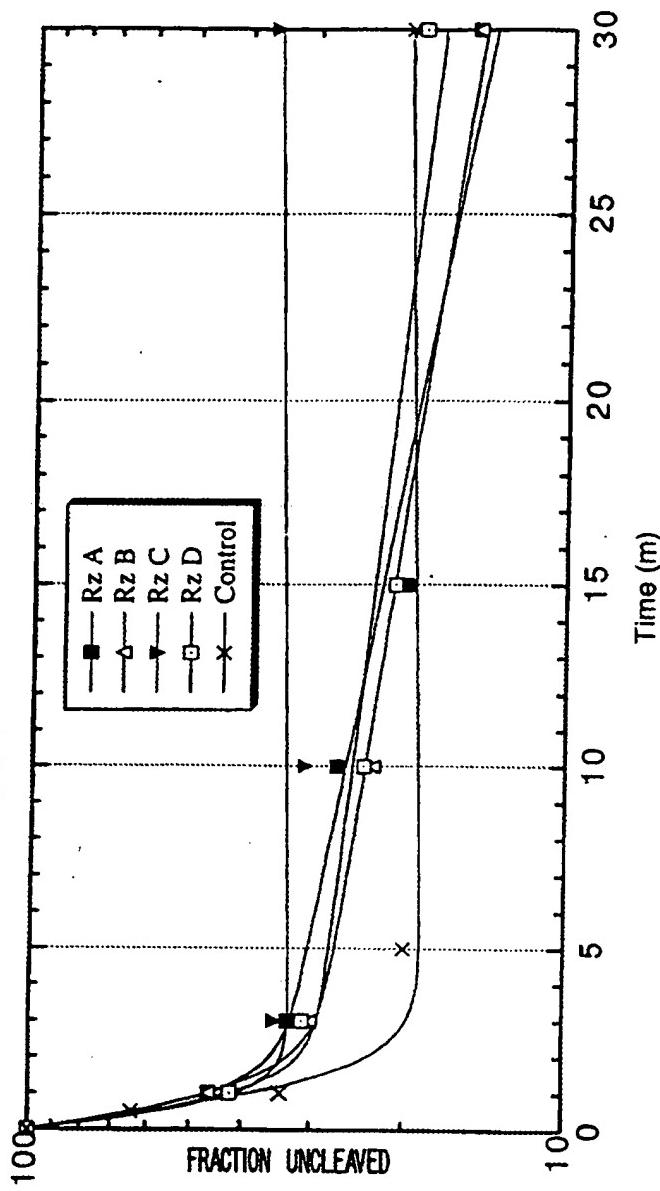
$Rz B$		
$y = m1 \cdot \exp(-m2 \cdot m0) + m3 \cdot \exp(-m4 \cdot m0)$		
	Value	Error
m1	20.898	14.545
m2	0.21686	0.1902
m3	80.591	14.99
m4	0.015244	0.0072584
Chisq	28.467	NA
R	0.99282	NA

$Rz C$		
$y = m1 \cdot \exp(-m2 \cdot m0) + m3 \cdot \exp(-m4 \cdot m0)$		
	Value	Error
m1	24.205	3.1876
m2	1.2349	0.40326
m3	75.913	2.6166
m4	0.022247	0.0039455
Chisq	7.7965	NA
R	0.99665	NA

$Rz D$		
$y = m1 \cdot \exp(-m2 \cdot m0) + m3 \cdot \exp(-m4 \cdot m0)$		
	Value	Error
m1	38.104	6.7642
m2	0.40912	0.13303
m3	60.267	6.6808
m4	0.0074357	0.0019864
Chisq	27.831	NA
R	0.99255	NA

$Rz E$		
$y = m1 \cdot \exp(-m2 \cdot m0) + m3 \cdot \exp(-m4 \cdot m0)$		
	Value	Error
m1	1.9896	1.9896
m2	0.50395	0.060309
m3	66.98	1.9005
m4	0.0065002	0.0013287
Chisq	3.2535	NA
R	0.99888	NA

FIG. 8b.



$Rz\ A$		
	Value	Error
m1	64.468	3.0502
m2	1.7159	0.23412
m3	35.528	2.4005
m4	0.03126	0.0052961
Chisq	7.2589	NA
R	0.999926	NA

$Rz\ B$		
	Value	Error
m1	69.397	1.3705
m2	1.45	0.075309
m3	30.585	1.081
m4	0.024723	0.0025193
Chisq	1.4949	NA
R	0.99985	NA

$Rz\ C$		
	Value	Error
m1	67.378	4.4664
m2	2.0834	0.5058
m3	32.614	3.0583
m4	-0.0027211	0.0048674
Chisq	10.656	NA
R	0.99841	NA

$Rz\ D$		
	Value	Error
m1	69.572	3.5965
m2	1.7304	0.2666
m3	30.408	2.693
m4	0.01864	0.0060488
Chisq	11.594	NA
R	0.99878	NA

$Rz\ E$		
	Value	Error
m1	82.852	10.324
m2	1.4042	0.36303
m3	18.289	8.1056
m4	-0.0032029	0.0199
Chisq	46.535	NA
R	0.99506	NA